Influence of soil type and natural Zn chelates on flax response, tensile properties and soil Zn availability

Jose M. Alvarez

Abstract A greenhouse experiment was conducted on weakly acidic and calcareous soils to evaluate the relative efficiencies of three natural Zn chelates [Zn-aminelignosulphonate (Zn-AML), Zn-polyhydroxyphenylcarboxylate (Zn-PHP) and Zn-S, S-ethylenediaminedisuccinate (Zn-S,S-EDDS)] applied to a crop textile flax (*Linum ussitatisimum* L.) at application rates of 0, 5 and 10 mg Zn kg^{-1}. In the flax plant, the following parameters were determined: dry matter yield, soluble and total Zn concentrations in leaf and stem, chlorophyll, crude fibre, and tensile properties. For the different soil samples, the following parameters were determined: available Zn (DTPA-AB and Mehlich-3 extractable Zn), easily leachable Zn ($BaCl_2$-extractable Zn), the distribution of Zn fractions, pH and redox potential. On the basis of the use of added Zn by flax, or Zn utilization, it would seem recommendable to apply Zn-S,S-EDDS at the low Zn rate in both soils. In contrast, adding the high Zn rate of this chelate to the weakly acidic soil produced an excessive Zn concentration in the plant, which caused a significant decrease in both dry matter yield and chlorophyll content. Furthermore, assessing available Zn with the DTPA-AB method proved the best way of estimating the level of excess Zn in flax plants. The soluble Zn concentration, which was established with 2-(*N*-morpholino)ethanesulfonic acid reagent (MES), of plant fresh and dry matter could be used as an alternative way of diagnosing the nutritional status of Zn in flax plants. In this experiment, the highest soil pHs were associated with the lowest redox potentials, which coincided with the smallest amounts of available Zn and water soluble Zn in soil, and the lowest levels of Zn uptake by flax plants.

Keywords Bioavailability · Chlorophyll · Crude fibre · Fertilizer · Flax · pH · Redox potential · Soil Zn status · Tensile properties · Zn chelates

Abbreviations

AB	Ammonium bicarbonate
AMOX	Amorphous Fe oxides
CAR	Carbonate
CRYOX	Crystalline Fe oxides
DM	Dry matter
DTPA	Diethylenetriaminepentaacetic acid
EXC	Exchangeable
FM	Fresh matter
MnOX	Mn oxides
OM	Oxidizable (organic material and sulphide)
RES	Residual

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WS	Water soluble
Zn-AML	Zn-aminelignosulphonate
Zn-PHP	Zn-polyhydroxyphenylcarboxylate
Zn-S,S-EDDS	Zn-S,S-ethylenediaminedisuccinate

Introduction

Zinc is an essential element for normal crop growth and Zn deficiencies can severely impair crops and reduce yields (Brennan et al. 1993; Fageria et al. 2002; Gangloff et al. 2002). The potential for Zn deficiencies is greatest in soils with low organic matter contents and pH levels above 7.0. In these situations, Zn deficiencies are easily corrected by applying fertilizers that contain high quantities of water-soluble Zn, such as organic Zn complexes or Zn chelates (Cakmak 2008; Alloway 2008). Now that Zn fertilization has become a common agricultural practice, it is foreseeable that, with time, many agricultural soils will receive Zn applications in excess of what is needed to correct Zn deficiencies. To prepare appropriate applications for different plant cultures, more information is required on the tolerance of the various plant species (Paschke et al. 2006) to excessive levels of available Zn in the soil. Several cases of Zn toxicity, affecting alfalfa, tomato, lettuce, onion, spinach, pea, soybean and corn, have been reported in the literature (Boawn and Rasmussen 1971; Vitosh et al. 1994). In general, reports of Zn toxicity affecting plants have involved acidic soils or solution cultures.

Flax (*Linum ussitatisimum* L.) is an economically important oilseed crop. It is cultivated for its fibre and oil (Herdrich 2001; Maherani et al. 2007). Zinc deficiency is expressed as a condition known as "chlorotic dieback". Plants affected by Zn deficiency tend to be pale in colour and their growing point may die. Later in the season, the plants may sprout new shoots from lower nodes and form a candelabra appearance and maturity may be delayed (Franzen 2004; Moraghan 1978). Flax is a plant that is very susceptible to Zn deficiency, particularly at low soil temperatures (Moraghan 1980). This susceptibility is heightened by P application (Burleson and Page 1967; Jiao et al. 2007; Loneragan et al. 1979; Millikan et al. 1968; Moraghan 1984; Spratt and Smid 1978) and also influenced by the presence of Fe, which determines normal chlorophyll formation and the development of green plant tissue (Lee et al. 1969). Field studies have been conducted to evaluate the interactive effects of phosphate and $ZnSO_4$ fertilizers on different soil types and Cd uptake and their distribution in flax (Grant and Bailey 1997; Jiao et al. 2004). Various abnormalities, which are apparently nutritional in origin, often retard growth early in the growing season (Flor 1943; Fieldes and Gerhardt 1994). According to Moraghan (1993), Zn deficiency delays flowering and boll formation by up to 20 days and reduces seed size, while soil-applied $ZnSO_4$ may reduce Cd concentration in flax. Grant et al. (2000) reported that environment, cultivar and fertilizer management are the main factors controlling Zn and Cd uptake by flax. Other elements, such as Ca or Mg, can be related to a yield response to Zn applied to flax crops in the form of $ZnSO_4$, the exact nature depending on soil type (Grant and Bailey 1989). Numerous researchers have reported that the application of Zn fertilizers repaired Zn deficiencies in plants (Kádár et al. 2003; Mortvedt and Gilkes 1993; Prasad and Sinha 1981), but few have compared the relative efficiency of applying different Zn sources to flax crops or its influence on the different mechanical properties of flax.

Many claims have been made regarding the relative efficiency of organic vs. inorganic ($ZnSO_4$) Zn sources (Gangloff et al. 2002). Producers of organic Zn sources generally claim that their products are up to ten times more efficient at satisfying agronomic demands than their inorganic counterparts, but some researchers have reported that the real advantage of applying "true" Zn chelates (such as Zn-EDTA) is in the order of 3–5 times. The effectiveness of the synthetic Zn chelates is, however, reportedly different for various chelating agents (Alvarez 2007). According to Luo et al. (2006) and Nörtemann (2005), aminepolycarboxylate chelating agents, similar to EDDS, that form complexes with either relatively low or moderately high stability constants are readily degradable, while those that form stronger complexes are resistant to biodegradation. As these natural Zn chelates are biodegraded in the soil, there is a reduction in the availability of Zn and its effects on plants consequently diminish, but the exact nature of this relationship can depended on the particular characteristics of the soil and particular chelating agent (Alvarez et al. 2009). Soil parameters

such as pH and redox potential can affect the behaviour of the natural organic Zn chelates and modify their potential bioavailability (Charlatchka and Cambier 2000; James and Bartlett 2000; McBride 1989; Patrick et al. 1996; Sajwan and Lindsay 1988).

The speciation of metal plays a fundamental role in the way in which transfers between different soil compartments take place and soil minerals migrate towards plants (Chandi and Takkar 1982; Mench and Martin 1991). However, the mechanism by which chemical reagents are selected has remained a question for debate and so, despite certain limitations, sequential extraction is still generally considered the best way to evaluate the different forms and associations of metals present in soil (Alvarez et al. 2006; Ure 1995). Another approach for estimating the availability of metals to plants using single extractions such as DTPA-ammonium bicarbonate (DTPA-AB) and Mehlich-3 methods, which tend to correlate well with metal concentrations in plants (Adriano 2001; Jones 2001).

The objective of this study was to determine the influence of three commercial Zn chelates of natural origin on responses by textile flax grown in two types of soil under greenhouse conditions. Extraction procedures were applied to reveal the Zn distribution in soil fractions and Zn bioavailability in soils. pH and redox potential were also evaluated in the course of the experimentation. The effectiveness of Zn sources was assessed in terms of the dry matter yield of the flax crop and its total and soluble Zn concentrations, chlorophyll and crude fibre contents, and tensile properties.

Materials and methods

Soil characterization

The two soils used in this study were surface horizons (A_p horizons) and came from two different regions of Spain. The soil called Soil I was from Madrid (latitude 40°17′ N, longitude 4°01′ W) and the soil denominated Soil II was from Guadalajara (latitude 40°39′ N, longitude 3°19′59″ W). Soil I was classified as a Typic Haploxeralf and Soil II as a Typic Calcixerept (Soil Survey Staff 2006). The two soils were previously cultivated with cereals or vines. Samples of the two soils were air-dried and sieved at 2 mm, with the fraction passed through the sieve being used in this study. Some properties of the two soils are shown in Table 1. Particle-size analysis was carried out according to the hydrometer method (Day 1965) and the soil textures (USDA) were sandy loam and loamy sand, respectively. The predominant clay in Soil I was illite, whereas in Soil II, it was smectite (X-ray diffraction technique; Schultz 1964). Water-holding capacity was measured by the procedure described by Klute (1996). Soil pH, redox potential (E_h) and electrical conductivity were measured in deionized water at a 1:2.5 (w:v) soil:water ratio (Chapman and Pratt 1961; ISO 11271 2002). Extractable P in Soil I was extracted with the Bray-1 extractant (Bray and Kurtz 1945, suitable for acidic soil) and the Olsen extraction procedure was applied to Soil II (Olsen et al. 1954, suitable for alkaline soil). Organic matter was measured by the Walkley-Black

Table 1 Selected soil properties used in this study

Soil properties	Soil I	Soil II
Sand (g kg^{-1})	840	560
Silt (g kg^{-1})	60	260
Clay (g kg^{-1})	100	180
Water-holding capacity (33 kPa, g H_2O 100 g^{-1} soil)	6.60	20.5
pH	6.13	8.13
Redox potential (mV)	458	380
Electrical conductivity (μS cm^{-1})	37.2	178
Extractable P (mg kg^{-1})	19.9	12.6
Oxidizable OM (g kg^{-1})	5.00	12.9
Total N (g kg^{-1})	1.00	1.10
Cation exchange capacity ($cmol_c$ kg^{-1})	4.72	23.5
Fe (active Fe_2O_3) (mg kg^{-1})	141	56
Total Zn (mg kg^{-1})	9.97	44.25

procedure (Hesse 1971) and total N was determined by Kjeldhal digestion (Bremner 1996). In Soil II (calcareous), total and free $CaCO_3$ contents were measured with a Bernard calcimeter (Allison and Moodie 1965; Nijensohn and Pizarro 1960), with the values determined being 134 and 33 g kg^{-1} respectively. Cation exchange capacity was assessed with both sodium acetate (NaOAc, pH 8.2) and ammonium acetate (NH_4OAc, pH 7.0) extractants (Bower et al. 1952). Active Fe (in poorly crystalline oxides) was extracted with dithionite-citrate-bicarbonate buffered at pH 7.0 (McKeague and Day 1966). Total soil Zn was determined by digesting a 1 g sample with 14 mL of HNO_3 (65%) and 6 mL of HF (35%) in Teflon vessels in a microwave oven (CEM Corporation, model-Mars, Matthews, NC, USA) in a two-step process (maximum pressure 690 kPa) (see the total Zn concentrations of the original soils in Table 1).

Fertilizers applied

The liquid fertilizers used were: Zn-aminelignosulphonate (Zn-AML) (Zn-Poli-Green product), Zn-polyhydroxyphenylcarboxylate (Zn-PHP) (Zn-Long product) and Zn-S,S′-ethylenediaminedisuccinate (Zn-S,S-EDDS) (Zn-6-King-Biological product). These fertilizers are marketed by several different companies and have Zn concentrations (*w*/*w*) of 3.3% (mass density = 1.20 g cm^{-3}), 6.0% (mass density = 1.26 g cm^{-3}) and 3.0% (mass density = 1.36 g cm^{-3}), respectively (Liñán 2008).

Greenhouse pot experiment

14 kg of air-dried soil were placed in polyethylene pots (or containers, each with a capacity of 15 L, internal diameter of 26.5 cm and height of 25 cm) and kept in a greenhouse. The bottoms of the pots were sealed so that the experiment was conducted without any leaching. These pots of soil were fertilized with 100 mg N kg^{-1}, which was applied in two separate doses (the first at sowing and the second 45 days after sowing) in the form of $(NH_2)_2CO$; 120 mg P kg^{-1}, in the form of KH_2PO_4; and 150 mg K kg^{-1}, in the form of KH_2PO_4 and K_2SO_4.

The plant used in this study was the NATASJA (AGROSA S.A., Guadalajara, Spain) variety of textile-producing flax. One hundred and twenty flax seeds were sown in each pot at a depth of 3 cm. After germination, seedlings were removed so that only 40 were left in each pot. The control treatment (with no Zn addition) and fertilizer treatments (5 and 10 mg Zn kg^{-1}) were replicated 3 times for each soil according to a randomized complete block design (total pot number: 42).

The pots were irrigated with appropriate amounts of potable water to reach and approximately maintain conditions to 75% of normal field capacity. To evaluate evapotranspiration, the containers were weighed (balance A&D Instruments Ltd., UK, model FG-30 KBM) and the volume of irrigation water required was estimated. During the experiment, the upper part of the soil was turned 6 times to facilitate aeration. The greenhouse temperature varied from 10°C to 32°C and relative air humidity ranged from 60% to 85%. The experiment was performed in spring (from 19 March to 17 June) with high light intensities. The plants reached an approximately height of 90 cm in Soil I and 80 cm in Soil II.

Chemical analysis of the plants

While the flax was in the pot (just before harvest), samples of leaves were collected to analyze both soluble Zn in fresh matter (FM), by means of an extraction with 2-(*N*-morpholino)ethanesulfonic acid (1 mM at pH = 6.0, called MES reagent) (Rahimi and Schropp 1984; Cakmak and Marschner 1987), and chlorophyll content. Soluble Zn in fresh leaf was determined by MES reagent after collecting 0.5 g of leaves from between the seventh and fifteenth leaf layers in the upper part of the plant. These leaves were manually macerated in a mortar with 10 mL MES reagent for approximately 5 min. Later they were centrifuged (10,000 rpm for 15 min) and filtered through Whatman n° 41 and then Zn was determined in the supernatant solution. The AOAC Official Methods of Analysis (AOAC 942.04, 1990) procedure was followed to determine the a, b and total chlorophyll contents of the leaves. 0.2 g fresh leaf was then macerated in a mortar with acetone (85%) until the leaves were a whitish colour. The resulting suspension was then filtered through Whatman n° 41 in a 50 mL flask covered with aluminium paper. The level of absorbance (A) at each wavelength was measured in a UV-1603 spectrometer (Shimadzu)

and chlorophyll content was quantified by applying the following equations: a chlorophyll = $9.93\,A_{660.0\,nm} - 0.777A_{642.5\,nm}$; b chlorophyll = $17.6\,A_{642.5\,nm} - 2.81\,A_{660.0\,nm}$; total chlorophyll = $7.12\,A_{660\,nm} + 16.8\,A_{642.5\,nm}$.

Ninety days after sowing, and without any significant seed development, the plants were cut at soil level, washed twice with deionized water, dried and then kept in paper bags. They were subsequently dry to a constant weight in a forced-draft oven at 60°C. The dried matter yield was then determined by weight. Stems and leaves were separated, ground and kept in sealed containers for later analysis. 0.25 g subsamples of stems and leaves were subjected to wet digestion in a microwave oven (maximum pressure 1,100 kPa) using an acid mixture [5 mL of HNO_3 (65%) + 2 mL of HF (65%)]. The rest of this procedure was the same as that employed with the soil samples.

Soluble Zn in dry leaf was determined by extracting 0.25 g ground dry leaf with 10 mL MES reagent for 5 h through rotative agitation at 120 rpm. The leaf-extractant suspension was filtered with Whatman n° 41 and Zn concentrations were determined by flame atomic absorption spectrophotometry (FAAS) (Perkin-Elmer AAnalyst 700).

Crude fibre content (% in dry matter) was determined by the Weende method (AOAC 985.29, 1990), using Fibertec System M2 equipment (Tecator, Höganäs, Sweden). Crude fibre was analyzed by weighing 1 g of ground dry stem in a 30 mL glass crucible and then submitting the fibre to extraction with 150 mL of 0.128 M H_2SO_4 and 150 mL of 0.223 M KOH. The digested samples were dried overnight at 100°C in a forced-draft oven and converted into ash by heating at 500°C for 3 h in a muffle furnace, but they only provided an ash content <0.001 g.

Plant mechanical properties

Flax is characterized by its tensile properties, including its tensile strength, Young's modulus and elongation at break (Kirkham 2005; Mohanty et al. 2005). Tensile strength is the stress needed to break a sample (where stress is the force applied per unit of cross-sectional area of the material: stress = F/A) and is usually expressed in MPa. Young's modulus (the modulus of elasticity: E) is the ratio of stress to strain (where strain can be defined as the ratio of change in length that results from stress being applied to the original length of the material: strain = ΔL/L) and is usually expressed in GPa. This modulus can be experimentally determined from the slope of a stress-strain curve. Elongation at break (ultimate elongation) is the strain placed on a sample when it breaks and is usually expressed as a percentage.

Five stems from each pot were cut to similar lengths (a 12.0 cm length was clipped from the middle of the stem), and their diameters were measured with a slide gauge and ranged from 1.0 to 2.4 mm. Tensile tests were carried out using an instrument for testing different materials (Texture Analyzer XT2) and tensile properties were determined using Texture Expert Software (Texture Technologies Corp., Scarsdale, NY, 2005).

Soil chemical analysis

The concentration of Zn available for plants was determined with DTPA-AB [0.005 M DTPA + 1.0 M NH_4HCO_3 adjusted to pH 7.6 with NH_3 (aq.)] (Soltanpour 1991) and Mehlich-3 (0.2 MHOAc + 0.25 MNH_4NO_3 + 0.015MNH_4F + 0.013 $MHNO_3$ + 0.001 MEDTA) (Mehlich 1984). Easily leachable Zn was extracted with the $BaCl_2$ reagent according to Schultz et al. (2004), which is a modified version of Normal ISO 11260 (1994). This procedure consisted of weighing a 3 g soil sample in a 125 mL conical glass flask and extracting the leachable Zn with 30 mL of 0.01 M $BaCl_2$ reagent for 2 h at 260 rpm. The soil suspension was centrifuged (4,000 rpm for 10 min) and its pH was measured. Next, the supernatant was filtered by vacuum pump, with 0.45 μm cellulose acetate membrane filter (Albet 47BL, Barcelone, Spain).

The partitioning of metal into the component parts of the soil solid-phases was investigated by selective sequential extraction (SSE). The SSE used was an eight-step procedure and was performed with 2.5 g of dry soil, according to slightly modified versions of methodologies used by different authors (Becquer et al. 2006; Leleyter and Probst 1999; Quantin et al. 2002) (Table 2). The geochemical compartments targeted were water soluble (WS), exchangeable (EXC), carbonate bound (CAR), Mn oxide bound (MnOX), amorphous Fe oxide bound (AMOX),

Table 2 Selective sequential extraction (SSE) procedure of Zn from soil

Step	Fraction	Extraction solution	Volume (mL) or weight (g)	Conditions
1	Water soluble (WS)	Deionized water	25 mL	Sake 30 min
2	Exchangeable (EXC)	1 M $Mg(NO_3)_2$ (pH 5)	25 mL	Shake 2 h
3	Carbonate bound (CAR)	1 M $NaCOOCH_3$	25 mL	Shake 4 h
4	Mn oxide bound (MnOX)	0.1 M $NH_2OH{\cdot}HCl$	25 mL	Shake 30 min
5	Amorphous Fe oxide bound (AMOX)	0.2 M $(NH_4)_2C_2O_4{\cdot}H_2O$ + 0.2 M $H_2C_2O_4$ (pH 3)	25 mL	Shake 4 h (in dark)
6	Crystalline Fe oxide bound (CRYOX)	(1) CB: $Na_3C_6H_5O_7{\cdot}2H_2O$ (78.4 g/L) + $NaHCO_3$ (9.82 g/L) (2) $Na_2S_2O_4$ (pH 7)	(1) 25 mL CB. (2) 1 g $Na_2S_2O_4$	80°C, 15 min in CB and 30 min after adding $Na_2S_2O_4$ and shaking
7	Oxidizable (organic material and sulphide bound) (OM)	(1) 0.02 M HNO_3–35% H_2O_2 (2) 3.2 M NH_4COOCH_3 (20% *v*/*v* HNO_3)	(1) 3–6 mL (2) 10 mL	(1) 85°C, 5 h (2) 85°C, 30 min
8	Residual (RES)	Differences between the total Zn and the sum of the previous fractions	–	–

CAR fraction (step 3) only was determined in Soil II (calcareous)

crystalline Fe oxide bound (CRYOX), oxidizable (OM) and residual (RES). The concentration of residual Zn was calculated by subtracting the other fractions from total Zn.

"Perkin-Elmer Pure" standard checks were used for the Quality Assurance System (certified by NIST-SRM). Standard solutions of Zn were prepared for each extraction in a background solution of the extracting agents. In all cases, Zn concentrations were determined by FAAS.

Statistical analyses

Multiple comparisons of variables were made using the LSD separations of means procedure. Simple regression analysis techniques were also applied. All analyses were performed using the Statgraphics Plus-5.1 software (Manugistic Inc., Rockville, MD, USA) and a probability level of $P \leq 0.05$ was selected to establish statistical significance.

Results

Flax response to zinc fertilization

The effect of fertilizer treatments on flax dry matter yield and Zn concentration (soluble and total) in harvest plants is shown in Table 3. The different concentrations of Zn obtained from leaves and whole shoots for all treatments were greater for Soil I than for Soil II and the Zn- S,S-EDDS chelate (at both Zn rates) caused the highest plant concentrations in both soils. The concentrations of soluble Zn in fresh matter (FM) from leaves at the high rate of Zn-S,S-EDDS chelate application (10 mg Zn kg^{-1}) were 7 times greater than for the control treatment (no Zn addition) in soil I and 5.3 times greater in soil II. The low rate of this Zn source (5 mg Zn kg^{-1}) also produced greater micronutrient concentrations than the other two sources. The two high rates of Zn-AML and Zn-PHP produced maximum values that were zrespectively only 3.3 and 2.8 times greater than the control treatment in Soil I and 2.2 and 1.6 times greater in Soil II. Concentrations of soluble Zn in dry matter (DM) from leaves followed a similar trend, but in this case the high rate of Zn-S,S-EDDS produced values that were respectively 4.9 and 3.7 times greater than the controls in soils I and II. In the most favourable cases of the other two sources (at the high rate of Zn-AML), values were only respectively 2.5 and 1.5 times greater than for the control concentrations in soils I and II.

In Soil I, only high-rate applications of the Zn-S,S-EDDS chelate produced a significant decrease in dry matter yield, with values 1.5 times lower than for the control treatment (see Table 3, "a" group). In Soil II, all the Zn treatments produced increases in plant dry

Table 3 Effect of Zn fertilization with 5 and 10 mg Zn kg^{-1} soil as Zn-aminelignosulphonate (Zn-AML), Zn-polyhydroxyphenylcarboxilate (Zn-PHP) and Zn-S,S′-ethylenediaminedisuccinate (Zn-S,S-EDDS) in the response of the flax crop

Treatment	Zn rate (mg kg^{-1})	Soluble Zn conc. FM of leaves (mg kg^{-1})	Soluble Zn conc. DM of leaves (mg kg^{-1})	Dry matter yield (g pot^{-1})	Total Zn conc. DM of whole shoots (mg kg^{-1})	Zn uptake (mg pot^{-1})	Zn utilization (%)
Soil I							
Control	0	6.37a	58.55a	19.62b	42.71a	0.838	–
Zn-AML	5	12.19b	92.95ab	23.56b–d	86.05c	2.027	1.70
	10	21.31c	147.50c	24.97b–d	129.67e	3.238	1.71
Zn-PHP	5	11.79b	91.05ab	26.11 cd	70.40b	1.838	1.43
	10	17.68c	125.92bc	26.83d	96.02c	2.576	1.24
Zn-S,S-EDDS	5	30.69d	203.09d	20.74bc	115.56d	2.397	2.23
	10	44.94e	287.86e	12.75a	172.12f	2.195	0.97
Soil II							
Control	0	4.46a	47.84[a]	12.60a	22.99a	0.290	–
Zn-AML	5	8.20c	65.65bc	17.15b	40.07bc	0.687	0.57
	10	9.76c	69.41c	16.83b	46.43c	0.781	0.35
Zn-PHP	5	5.00b	56.97b	16.44b	31.65ab	0.520	0.33
	10	6.94bc	64.35bc	15.31ab	37.28bc	0.571	0.20
Zn-S,S-EDDS	5	15.02d	127.68d	18.25b	92.05d	1.680	1.99
	10	23.66e	177.57e	17.78b	121.12e	2.154	1.33

For each soil, values compared using LSD multiple range test at the 0.05 level of probability. Homogeneous groups are denoted with the same letter

FM fresh matter; *DM* dry matter

matter yield with respect to the control, with a 1.4 time increase with respect to the control in the most favourable case (Zn- S,S-EDDS for 10 mg Zn kg^{-1}). Even so, this parameter did not increase when the application rate of each Zn source was doubled. As with Zn concentrations in leaves, the effect of Zn application on whole shoot Zn concentration was significant, particularly for Zn-S,S-EDDS in the two soils; maximum total concentrations reached 172 and 121 mg Zn kg^{-1}, respectively, from initial control levels of 43 and 23 mg Zn kg^{-1}, when Zn was not added to soils I and II (see Table 3). It was noticeable that when the control treatment was applied to Soil II, Zn tissue concentrations of almost 20 mg kg^{-1} were registered. Some authors (Alloway 2008; Jones 2001) cite this as the critical concentration for dried whole shoots of various different plants.

The values of total Zn concentrations in leaves and stems increased with the application of both dosages (Fig. 1), but especially when Zn-S,S-EDDS was used as the source of micronutrient. This was in line with findings reported by various authors, who state that applying EDDS increased plant micronutrient contents (Nörtemann 2005). When this chelate was applied to Soil I at the 10 mg Zn kg^{-1} rate, Zn concentrations in leaves and stems were 327 and 99 mg kg^{-1} DM, respectively: 4.9 and 3.2 times greater than in the control. In Soil II, the values obtained for these same treatments were 193 and 97 mg Zn kg^{-1} DM, respectively: 4.2 and 6.2 times greater than in the control. When Zn-AML was applied to Soil I, at both Zn rates, the Zn concentrations registered in leaves and stems were superior to those obtained with Zn-PHP treatments.

Potential availability, the distribution of zinc fractions and easily leachable zinc in soils

The distribution of the Zn fractions in the different soils (the total Zn concentration is the sum of the different fractions), available Zn (DTPA-AB- and Mehlich-3-extractable Zn) and easily leachable Zn ($BaCl_2$-extractable Zn) in the two soils are shown in Fig. 2. In Soil I and in the soil with no added Zn (control treatment), the Zn concentrations in the fractions decreased in the following order (mg kg^{-1}): RES (4.21), OM (1.50), EXC (1.13), MnOX (1.13), AMOX (0.97), CRYOX (0.80) and WS (0.21). In the soils treated with Zn, small differences in Zn distribution were observed for the three Zn chelates for each Zn rate. At 10 mg Zn kg^{-1}, the order of Zn distribution for the three fertilizers (mean values) was as follows: EXC (6.20), MnOX (3.75), RES (3.69), OM (1.92), AMOX (1.54), CRYOX (0.94) and WS (0.79). In this acidic soil, the Zn fertilizers therefore produced a significant increase in Zn content in fractions such as EXC and WS which could be considered very important for the Zn nutrition of the plant.

In Soil II, the Zn concentrations in the fractions for the control treatment decreased in the following order (mg kg^{-1}): RES (36.05), OM (3.85), CRYOX (1.53), AMOX (1.23), CAR (0.69), MnOX (0.38), EXC (0.28) and WS (0.10). According to the Zn rates applied, the orders of Zn distribution did not change in this soil for any of the three fertilizers (mean values) and at the application rate of 10 mg Zn kg^{-1}, the order was as follows: RES (39.85), OM (5.06), AMOX (3.25), CAR (2.67), CRYOX (1.90), MnOX (0.95), EXC (0.42) and WS (0.19). In this calcareous soil, the addition of the Zn fertilizes therefore produced only small increases in the most labile fractions (WS and EXC).

In Soil I, the available Zn concentrations (see Fig. 2) in the control soil (DTPA-AB- and Mehlich-3-extractable Zn: 1.20 and 1.89 mg Zn kg^{-1} respectively) were near what constitute critical levels for most plants (Soltanpour 1991; Tran and Shimard 1993). In the case of DTPA-AB-extractable Zn, the concentrations obtained for all the Zn treatments were higher than for the control. At the 5 mg Zn kg^{-1} rate, concentrations ranged from 3.0 to 3.7 times the control value, and at the 10 mg Zn kg^{-1} rate, they ranged from 6.7 to 9.2 times this value. Zinc extracted with $BaCl_2$ (easily leachable Zn) exhibited similar behaviour to available Zn. The Zn concentrations obtained at the 5 mg Zn kg^{-1} rate ranged from 2.2 to 2.5 times the control value, and those obtained at 10 mg Zn kg^{-1} ranged from 3.9 to 4.4 times the control value.

In Soil II, the available Zn concentrations in the control soil (DTPA-AB- and Mehlich-3-extractable Zn: 0.45 and 0.95 mg Zn kg^{-1}, respectively) were lower than those reported as critical for plants in calcareous soils. The concentrations obtained for the Zn-S,S-EDDS treatments were higher than for the other two fertilizers (considering that levels of DTPA-AB-extractable Zn applied at the 5 and 10 mg Zn kg^{-1} rates were respectively 6.0 and 12.1 times greater than

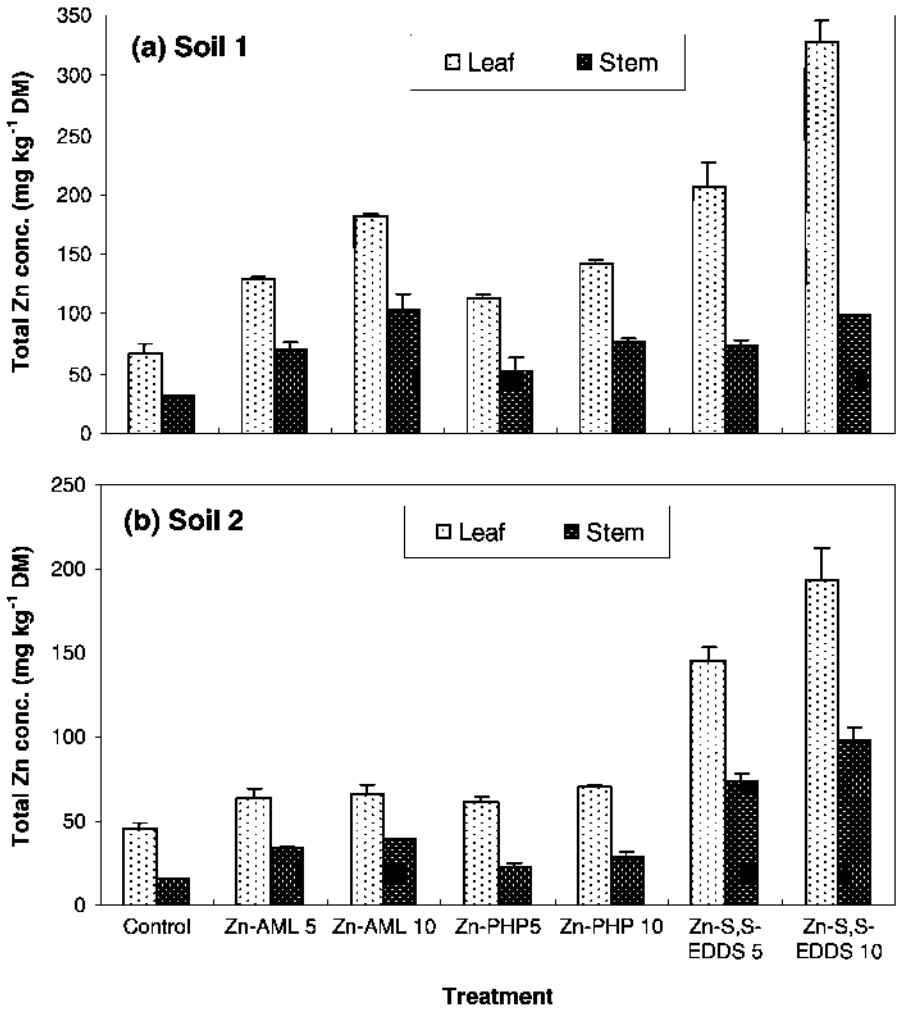

Fig. 1 Effect produced by the Zn treatments (5 and 10 mg Zn kg^{-1} soil as Zn-aminelignosulphonate (Zn-AML), Zn-polyhydroxyphenylcarboxylate (Zn-PHP) and Zn-S,S-EDDS] in Soil I (weakly acidic) (**a**, *top*) and Soil II (calcareous) (**b**, *bottom*) with respect to the total leaf and stem Zn concentrations in dry weight. *Vertical bar* at each of the data points represents the standard deviation from the mean of the concentration

the control). High Zn rates, applied as Zn-AML and Zn-PHP chelates, gave Zn concentrations that were only 3.8 and 6.7 times greater than the control value. For this calcareous soil, the $BaCl_2$ method extracted 0.13 mg Zn kg^{-1} with the control treatment, with maximum values of only 0.25 and 0.30 mg kg^{-1}, respectively, at the 5 and 10 mg Zn kg^{-1} rates. However, in both soils, and for all the fertilizer treatments, the amounts of easily leachable Zn were smaller than those obtained using the DTPA-AB and Mehlich-3 methods.

Zinc uptake per pot was positive and significantly correlated (for both soils, $n=14$) with the amounts of Zn extracted with the single soil extractants (DTPA-AB, Mehlich-3 and $BaCl_2$) ($P<0.001$ or <0.0001, r ranged from 0.79 to 0.87) and with the most labile Zn fractions (WS and EXC) ($P<0.001$, r ranged from 0.79 to 0.82). These high correlations indicated that Zn uptake by flax plants could be described as a function of easily soil-extractable Zn and, as a consequence of this, that Zn uptake depended on the capacity of natural Zn chelates to maintain Zn added as labile pools.

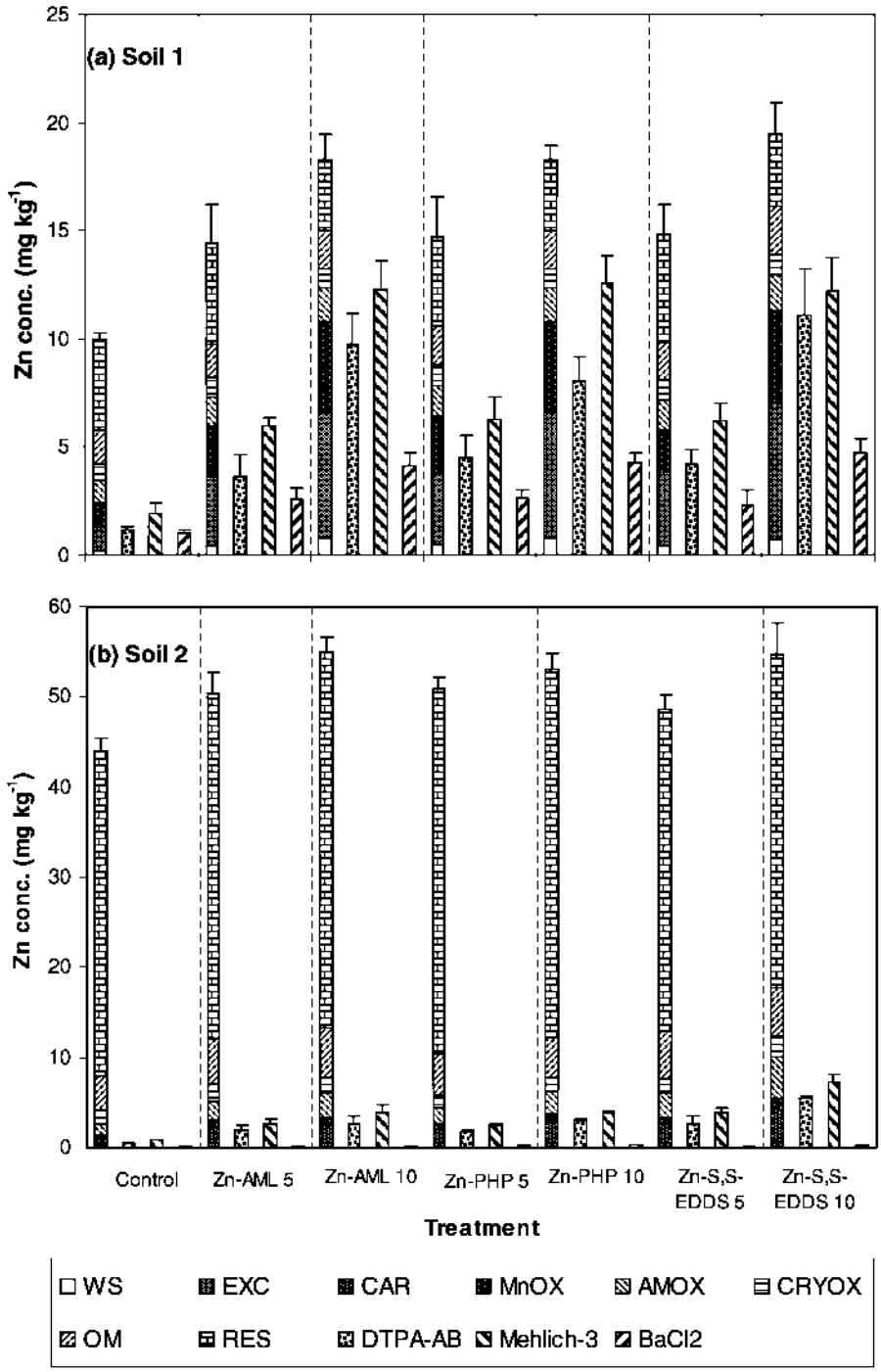

Fig. 2 Distribution of Zn fractions, DTPA-AB-, Mehlich-3-, and $BaCl_2$-extractable Zn in Soil I (weakly acidic) (**a**, *top*) and Soil II (calcareous) (**b**, *bottom*) with the fertilizer treatments at the moment of flax harvest. *Vertical bar* at each of the data points represents the standard deviation from the mean of the concentration

pH and redox potential parameters

pH and redox potential (E_h) values were measured in soils in a saturated paste at two different crop times: 45 and 90 d after seeding, for all treatments. These measurement points approximately corresponded to the half-way point and end of the experiment, respectively. For Soil I, the mean values

of the pH decreased with time, from 6.85 (at 45 d) to 5.65 (at 90 d) ($P<0.0001$) and there were no significant differences between different fertilizer treatments ($P>0.05$). In contrast, the E_h parameter significantly varied with the treatment factor ($P<0.05$) and the Zn-S,S-EDDS treatments were associated with smaller values (511 mV at the high Zn rate and 520 mV at the low Zn rate), but there were no significant differences associated with the time factor. The control E_h value obtained was 555 mV and the greatest value was 588 mV for Zn-AML at the low Zn rate. According to Lindsay (1979), the parameter pH + pe[pe = E_h(mV)/59.2], where pe is the negative logarithm of free electron activity, provides a convenient single-term expression for defining the redox status of soil systems. In this work, this parameter significantly varied with time ($P<0.0001$) from 16.10 (at 45 d) to 15.05 (at 90 d) and with the particular fertilizer treatment applied ($P<0.01$). The Zn-S,S-EDDS treatments were associated with smaller values (14.89 at the high Zn rate, 14.31 at the low Zn rate) while the control presented a value of 15.59 and Zn-PHP applied at the low Zn rate was associated with a value of 16.21, which was the greatest value.

In Soil II, the mean values of the pH decreased with time, from 7.93 (at 45 d) to 7.62 (at 90 d) ($P<0.0001$). As in Soil I, no significant differences were associated with fertilizer treatment factor. The E_h parameter significantly varied with the treatment ($P<0.01$) and greater values were obtained with the Zn-S,S-EDDS treatments (485 mV at the high Zn rate and 488 mV at the low Zn rate), while the lower value was 457 mV for Zn-AML at high Zn rate (control treatment, 467 mV). Significant differences were associated with the time factor ($P<0.001$), with values increasing from 463 mV (at 45 d) to 479 mV (at 90 d). In this calcareous soil, no significant differences were associated with the pH+pe parameter, according to the treatment factor [$P=0.052$; mean values ranged from 15.58 (control) to 16.05 (Zn-S,S-EDDS at the low Zn rate)] and time ($P>0.05$) factors.

The fact that the E_h (and also pe) values obtained for Soil I were higher than for Soil II, revealed more oxidizing conditions in Soil I than in Soil II. According to Patrick et al. (1996), the pH and pe values obtained in this study for both soils (pH+pe >14) corresponded to "oxic" or "normal" soils.

Chlorophyll, crude fibre and mechanical properties

The results for chlorophyll types with the fertilizer treatments registered in leaves from the flax plants at the end of experiment (90 d) are presented in Fig. 3. In Soil I, values of a, b, and total chlorophyll, showed significant differences between fertilizer treatments (P:<0.01, <0.05 and <0.01, respectively), with the Zn-S,S-EDDS treatments being associated with the lowest values (total chlorophyll: 1.99 mg g^{-1} for the low Zn rate and 1.50 mg g^{-1} for the high Zn rate). For the control treatment, the value obtained was 2.16 mg g^{-1} and the greatest value was 2.39 mg g^{-1} for Zn-AML applied at the low Zn rate. In Soil II, no significant differences between fertilizer treatments were noted for chlorophyll types. The a and b chlorophyll levels were greater in Soil I than in Soil II ($P<0.05$) (mean values of total chlorophyll: Soil I, 2.07; Soil II, 1.80).

In order to evaluate the behaviour of Zn sources, in this experiment crude fibre and three mechanical properties (tensile strength, Young's modulus and elongation at break) were determined in stem material (Fig. 4). As can be seen from the results for the application of Zn chelates, Zn-S,S-EDDS application at the 10 mg Zn kg^{-1} rate in Soil I, was associated with the lowest value for crude fibre (36%) obtained in all the treatments and with both soils, while the highest value was associated with Zn-PHP applied at 10 mg Zn kg^{-1} in Soil II (50%). Although no significant difference was obtained between the soils ($P=0.08$), mean values were 42% in Soil I and 45% in Soil II. Tensile strength values (MPa) ranged from 45 (Zn-AML applied at 10 mg Zn kg^{-1}) to 57 (Zn-PHP at 5 mg Zn kg^{-1}) in Soil I and from 50 (Zn-AML at 5 mg Zn kg^{-1}) to 83 (Zn-AML at 10 mg Zn kg^{-1}) in Soil II. For this last property, there were significant differences between the soils (at $P<0.0001$, the mean values for Soil I and II were 50 and 68 MPa, respectively). Likewise, for Young's modulus, the mean values for the two differed significantly (at $P<0.0001$, the mean values for soils I and II were 7.3 and 9.1 GPa, respectively) and the highest modulus obtained was for Zn-PHP applied at 10 mg Zn kg^{-1} (12 GPa) in Soil II. Finally, when the values were compared for the property elongation at break, it was observed that the two soils only differed significantly at $P<0.05$ (mean values for Soil I and II were 0.69% and 0.78%, respectively) and the highest value

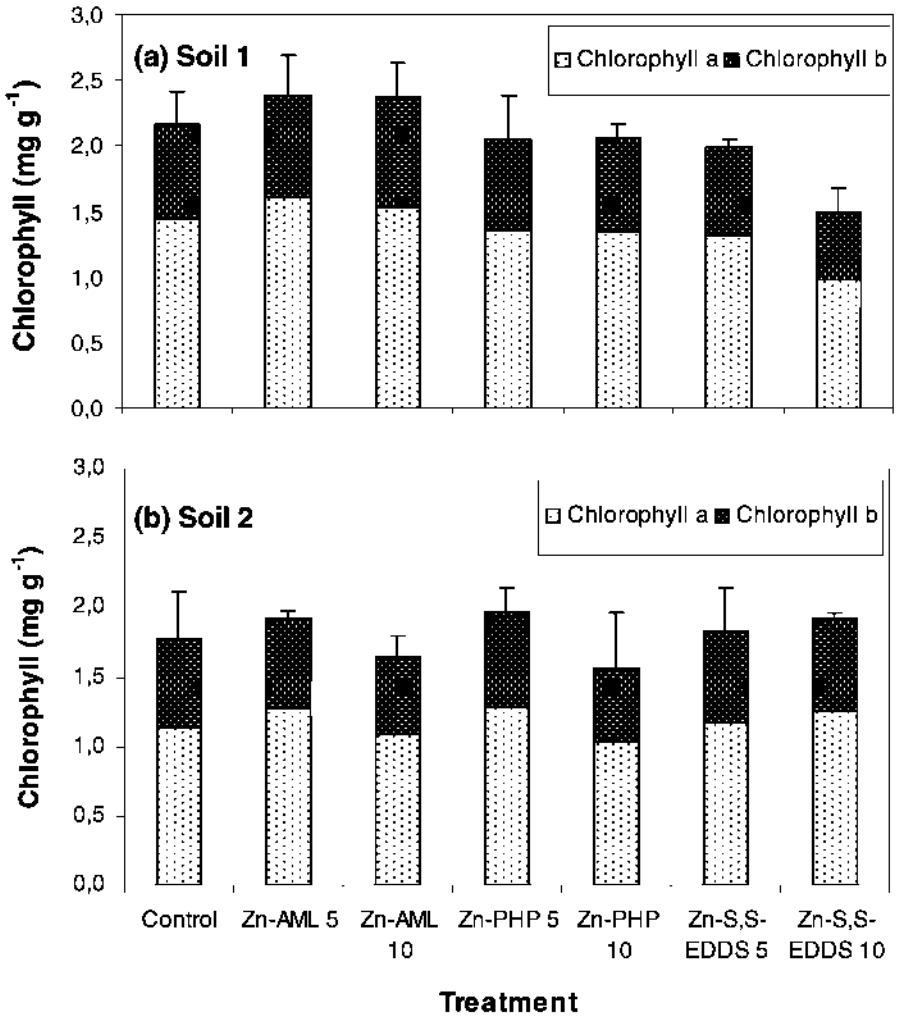

Fig. 3 Chlorophyll contents of fresh leaves from flax plants in Soil I (weakly acidic) (**a**, *top*) and Soil II (calcareous) (**b**, *bottom*) for the different fertilizer treatments at the end of experiment (90 d). *Vertical bar* at each of the data points represents the standard deviation from the mean for total chlorophyll

observed was for Zn-PHP, applied at 5 mg Zn kg^{-1} (0.90%) (see Fig. 4). According to Mohanty et al. (2005), for flax fibres with diameters of between 0.04 to 0.60 mm, the tensile strength can range from 345 to 1,500 MPa, the Young's modulus can be as high as 27.6 GPa and the elongation at break can range from 2.7% to 3.2%. In this study, which was carried out with large diameter fibres (see Materials and methods section), the values obtained for the tensile properties were smaller. This happened because it must be borne in mind that tensile properties follow a decreasing trend and scattering as a function of fibre diameter, also depends on which part of the stem (top, middle or bottom) is selected and on the length of the fibre studied. Joffe et al. (2003) indicated that Young's modulus needs to be plotted as a function of fibre diameter. Baley (2004) and Bos et al. (2006) reported that there is a correlation between tensile strength, Young's modulus and elongation at break, and increases in diameter. Charlet et al. (2007) presented the evolution of these properties of simple flax fibres according to their diameter and location on the stem. The values observed in this experiment performed in a greenhouse (based on the diameter of fibre used), were comparable with those obtained in other cases.

Discussion

In general, the Zn concentration in mature leaf tissue is sufficient or normal if it lies between 25 and 150 mg kg^{-1} (Kabata-Pendias and Mukherjee 2007)

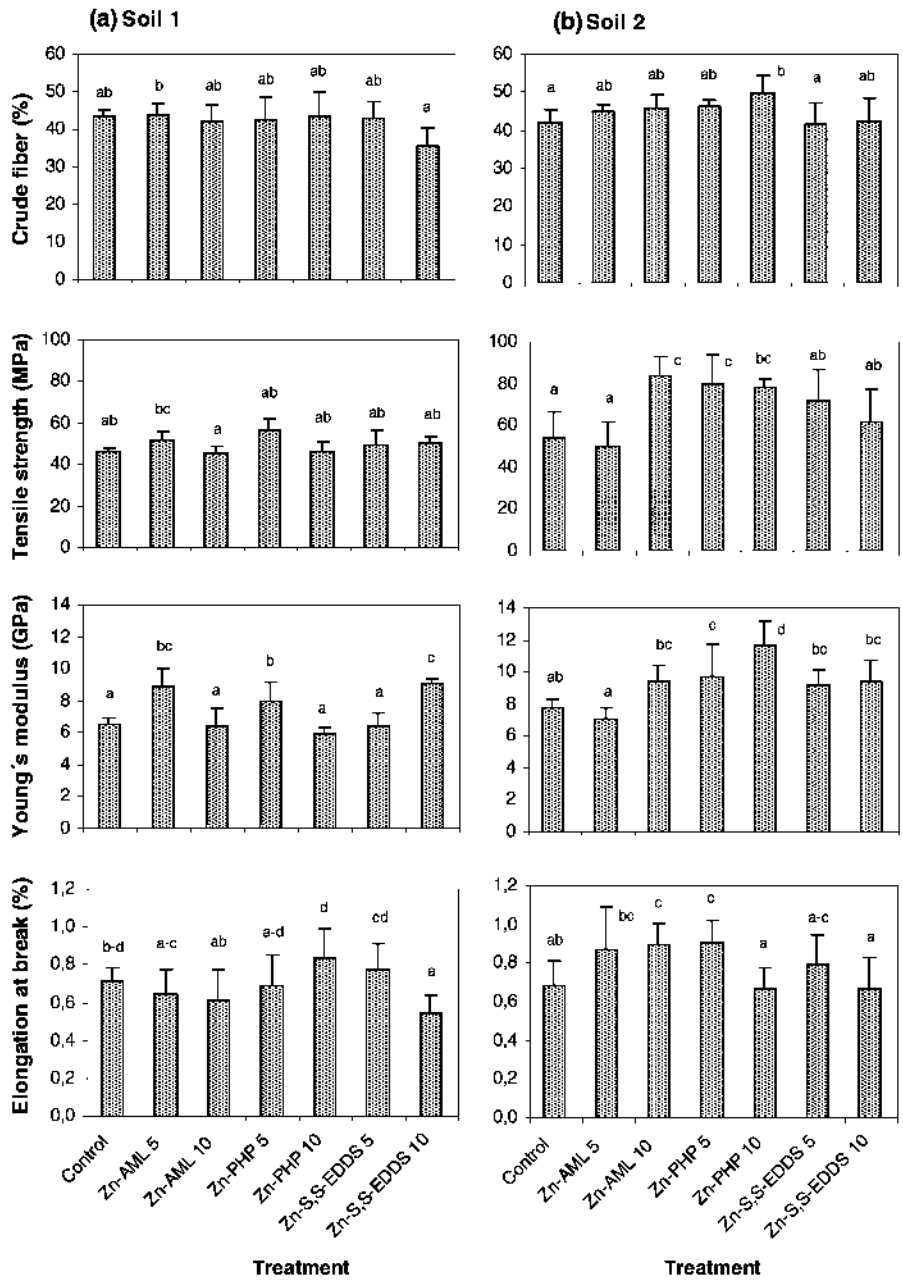

Fig. 4 Crude fibre, tensile strength, Young's modulus and elongation at break of the stems from flax plants in Soil I (weakly acidic) (**a**, *left column*) and Soil II (calcareous) (**b**, *right column*) with the fertilizer treatments at the end of experiment (90 d). *Vertical bar* at each of the data points represents the standard deviation from the mean

and is excessive or toxic at 300 mg Zn kg ^{1} or more (Vitosh et al. 1994). Average Zn concentrations in the plants in this study were higher in Soil I than in Soil II, with higher values for Zn-S,S-EDDS than for Zn-AML and Zn-PHP. The total Zn concentration DM in leaves only exceeded 300 mg Zn kg^{-1} at high rate of application of Zn in the Zn-S,S-EDDS chelate in Soil I; while the concentration of 150 mg Zn kg^{-1} was exceeded for Zn-AML in Soil I and for Zn-S,S-EDDS in Soil II (when applied at the high Zn rate in both cases). Zinc concentrations in stems were below 100 mg kg^{-1} and were generally less than half those found in leaves, being less than 25 mg Zn kg^{-1} in the case of Zn-PHP applied at the low Zn rate.

In Soil I, values of total Zn concentration in plant dry matter in all the Zn treatments were greater than those recommended by McDonald et al. (1981) as the lower limit of Zn in plants used for plant fodder

(50 mg kg^{-1}). In contrast, in Soil II, only the Zn-S,S-EDDS treatments produced total Zn concentrations that exceeded 50 mg kg^{-1}.

One important parameter in the study of the relative efficiency of any fertilizer is the percentage of its use by the crop. The percentage of Zn used by flax plants (Zn utilization) was defined according to the mode of application: [Zn uptake (fertilizer treatment)—Zn uptake (control treatment)] 100/Zn applied (see values in Table 3). In Soil I, the highest Zn percentages were obtained by applying the following fertilizer treatments: Zn-S,S-EDDS (5 mg Zn kg^{-1} rate) > Zn-AML (10 mg Zn kg^{-1} rate) ≈ Zn-AML (5 mg Zn kg^{-1} rate) > Zn-PHP (5 mg Zn kg^{-1} rate) > Zn-PHP (10 mg Zn kg^{-1} rate) > Zn-S,S-EDDS (10 mg Zn kg^{-1} rate). In other words, the greatest Zn use was associated with Zn-S,S-EDDS applied at the rate of 5 mg Zn kg^{-1}, which reached 2.23%. This was followed by Zn-AML treatments at both rates. Zinc applied with Zn-S,S-EDDS at a rate of 10 mg Zn kg^{-1} produced the lowest value, reaching only 0.97%. According to Schowanek et al. (1997) and Takahashi et al. (1997), the [S,S]-isomer is subject to rapid and complete mineralization. Over the last few years, the use of some biodegradable chelating agents, such as EDDS (S,S ethylenediaminedisuccinic acid), has been proposed as a way of enhancing the uptake of heavy metals (Luo et al. 2006; Grcman et al. 2001). In this weakly acidic soil (Soil I), the EDDS agent was more effective than the other sources applied and the high rate of Zn applied produced metal accumulation and toxicity in the flax plants. In this case, a concentration of 172 mg Zn kg^{-1} DM in whole shoots produced a significant reduction in Zn uptake by the flax crop. But this was the only case in which it was visually appreciated a decrease in the greenness of the flax plants and in which any of the plants died during the 90-day experiment.

In Soil II, the order of the Zn usage (%) was as the follows: Zn-S,S-EDDS (5 mg Zn kg^{-1} rate) > Zn-S,S-EDDS (10 mg Zn kg^{-1} rate) > Zn-AML (5 mg Zn kg^{-1} rate) ≈ Zn-AML (10 mg Zn kg^{-1} rate) > Zn-PHP (5 mg Zn kg^{-1} rate) > Zn-PHP (10 mg Zn kg^{-1} rate). In this soil, the amount of Zn used with Zn-S, S-EDDS, applied at the low Zn rate, was also greater than in the other treatments. In contrast, the Zn-PHP chelate was the least effective source since its Zn use reached only 0.33% at high rate of Zn application.

After applying Zn to Soil I (weakly acidic soil), higher Zn concentrations were registered for all the fractions in the treated soil than in the control soil (with no added Zn) (see Fig. 2). Applying the two Zn rates to this soil had a significant effect on its Zn content in the two most labile fractions: WS (from 5.6 to 5.8% of added Zn) and EXC (from 44% to 51% of added Zn). Even so, no significant differences were registered between the different fertilizer treatments, except in the case of the OM fraction, in which the highest Zn concentration was associated with Zn-S,S-EDDS at the high rate of Zn application (2.19 mg Zn kg^{-1}). Applying two different Zn rates to Soil II (calcareous) produced a considerable increase in Zn concentrations in the less potentially available fractions. The applied Zn therefore appeared to be predominantly associated with mineral lattices (RES ranged from 42% to 38% of added Zn), amorphous Fe oxides (AMOX ranged from 20% of added Zn) and carbonate (CAR ranged from 24% to 20% of added Zn), although levels of oxidizable fraction (organic material and sulphide bound) also increased (OM ranged from 23% to 12% of added Zn). The Zn contents of the two most labile fractions also increased, but only a little (WS+EXC ranged from 2.3% to 2.8% of added Zn). These calculations showed recoveries of Zn >100% with respect to applied Zn.

According to Franzen (2004), if soil Zn levels (DTPA-TEA-extractable Zn) are less than 1 mg Zn kg^{-1}, it is recommendable to add Zn. Various authors have reported that the amounts of Zn extracted with the DTPA-AB and Mehlich-3 methods are greater than those extracted with the DTPA-TEA method (Alvarez et al. 2009). In this study, and in both soils, all the Zn treatments produced values greater than the recommended level of available Zn. In Soil I, Zn-S,S-EDDS applied at the high Zn rate gave the highest concentration of available Zn (DTPA-AB-extractable Zn: 11.07 mg kg^{-1} and 57% of total Zn): a level that was excessive for flax plants. Easily leachable Zn exhibited a similar tendency, with the highest concentration being obtained with the same treatment (4.73 mg Zn kg^{-1}: 4.4 times the control value and 24% of total Zn). In Soil II, the highest concentration of available Zn was also obtained with this treatment (DTPA-AB-extractable Zn: 5.43 mg kg^{-1} and 10% of total Zn). The estimated maximum value for easily leachable Zn was 0.6% of the total Zn in this soil. Shuman (1991) reported that high clay and $CaCO_3$ contents cause

adsorption and the immobilization of added Zn. The alkaline pH probably made the Zn less soluble and fostered the formation of Zn hydroxides. Furthermore, the natural Zn chelates applied in this study were of medium or high stability and the Zn could partly be transferred from the chelates to soil binding sites. Finally, it is important to point out that in this study, the only DTPA-AB method correctly evaluated the excess pool of soluble or labile Zn (which is phytoavailable in weakly acidic soil) in relation to its phytotoxicity in flax plants when it was applied at the high rate of Zn- S,S-EDDS chelate application.

At the end of the experiment, the negative correlation between pH and E_h (and also pe) ($r=-0.93$, $n=14$) was very significant ($P<0.0001$). For a higher pH, we would therefore expect a lower E_h value. Soil pH showed a significant negative correlation with the WS, EXC and MnOX fractions ($P<0.01$) and a significant positive correlation with the AMOX, CRYOX, OM and RES fractions ($P<0.01$). According to this result, soil pH and the water soluble fraction of Zn showed a negative correlation and when soil pH increased, the availability of the three natural Zn chelates decreased.

In this experiment, the relationship between the total Zn concentration in leaf dry matter and the soluble Zn concentration extracted from leaf fresh and leaf dry matter, using the MES reagent ($r=0.98$ and $P<0.0001$ in both cases), indicated that MES soluble Zn content in the leaves of flax plants could be used to estimate the nutritional state of plants with respect to micronutrient. Independently of this result, determining the amount of soluble Zn by the methods proposed is easier than in the methods generally used to determine total or pseudo-total Zn. Furthermore, it does not require the use of strong acids.

Finally, Zn applied in form of Zn-S,S-EDDS was the source that produced the greatest amounts of Zn utilization by flax plants in both soils. Even so, in weakly acidic soils, is recommendable to apply this chelate at the low Zn rate, because the high Zn rate produced a decrease in dry matter yield. The behaviour of the other two chelates (Zn-amineligno-sulphonate and Zn-polyhydroxyphenylcarboxylate) was only satisfactory in the weakly acidic soil, particularly so in the case of the former, while increasing the Zn application rate with the two sources resulted in an increase in Zn uptake by flax plants.

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